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Processing and trafficking of clotting factor X in the secretory pathway

Effects of warfarin

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Clotting factor X undergoes several post-translational processing steps in the liver before the protein appears in blood as the mature two-chain zymogen. In this study we have followed the factor X precursor through the secretory pathway in rat liver in order to identify the site for proteolytic processing of the precursor into a two-chain form and the site for warfarin inhibition of precursor trafficking within the pathway. Isolated rat liver Golgi apparatus was shown to harbour two single-chains of factor X precursors of 70 and 74 kDa and the heavy (50 kDa) and light chains of factor X. It was demonstrated that the two-chain factor X form was produced from a late processing form of the factor X precursor, which indicated that the site for proteolytic conversion to a two-chain form was in the trans-Golgi compartment. The 70 and 74 kDa single-chain precursors and also the light chain of the two-chain form were shown to contain the factor X propeptide which is normally removed before the coagulation factor appears in blood. The data demonstrate that intra-chain cleavage of a single chain factor X precursor in the trans-Golgi compartment can precede release of the propeptide. Warfarin was shown to affect trafficking of the factor X precursor between the endoplasmic reticulum (ER) and the Golgi apparatus. The data suggest a link between vitamin K-dependent γ-carboxylation of the precursor and its exit from the ER. Warfarin administration resulted in accumulation of factor X precursors associated with the ER membrane. These precursors appear to be stabilized from intracellular degradation while in the ER. In contrast to the large increase in the factor X precursor concentration in the ER membrane, there was no change in the prothrombin precursor concentration as a result of warfarin action on the liver. However, intracellular turnover of the microsomal prothrombin precursor pool in warfarin-treated rats resulted in a pool of less negatively charged proteins, indicating ongoing protein synthesis but inhibition of γ-carboxylation. The data are consistent with previous findings [Wallin & Martin (1988) J. Biol. Chem. **263**, 9994–10001] suggesting that prothrombin and factor X are processed differently by the vitamin K-dependent carboxylase in the ER membrane.

INTRODUCTION

Vitamin K-dependent clotting factors are synthesized in the liver and appear in blood as zymogens of the coagulation system [1]. In the hepatocyte, newly synthesized precursor forms of these proteins are equipped with a signal peptide for translocation into the endoplasmic reticulum (ER) and a propeptide which links the signal peptide to the *N*-terminal part of the mature zymogen [2]. The signal peptide is cleaved by signal peptidase and the propeptide is the recognition element which destines these proteins for γ-carboxylation by the vitamin K-dependent carboxylase, an integral membrane protein of the ER [2,3]. The carboxylase requires reduced vitamin K, CO_2 and O_2 for the reaction, which converts 10–12 glutamic acid residues in the *N*-terminal part of the proteins to γ-carboxyglutamic acid (Gla) residues [2,4]. The modification is essential for optimal Ca^{2+}-mediated activation of the zymogens in blood [1].

On their 'journey' through the secretory pathway, the vitamin K-dependent proteins undergo additional modifications. All proteins acquire Asn-linked complex carbohydrate chains [5]. Protein C, protein S, factor VII, factor IX and factor X undergo Asp/Asn hydroxylation in their epidermal growth factor domain through the action of aspartyl β-hydroxylase [6]. All proteins undergo intracellular proteolytic processing which is demonstrated by the absence of the propeptide in the blood zymogens and the existence in blood of two-chain forms of the factor X and the protein C zymogens [2]. Recently our laboratory has shown that the prothrombin propeptide is cleaved by a Ca^{2+}-dependent serine proteinase located in the trans-Golgi compartment [7]. The enzyme appears to be similar to the mammalian KEX2-like gene product furin, which has been shown by Bresnahan *et al.* [8] to be involved in mammalian prohormone processing. Members of this KEX-2-like family of endoproteinases cleave many prohormones and precursors of secretory and membrane proteins at a pair of basic amino acids (Lys–Arg and Arg–Arg) [9,10]. Hosaka *et al.* [11] have provided data which support the notion that furin requires, in addition, a basic amino acid at position -4 for processing of secretory proteins within the constitutive pathway. Additional support for the involvement of the furin gene product in processing of vitamin K-dependent clotting factors has been provided by Foster *et al.* [12,13] through their studies on intracellular processing of protein C.

In this study we have followed the factor X precursor through the secretory pathway in order to identify both the site for proteolytic processing of factor X and the precursor form that is a substrate for the endoproteinase. We demonstrate the presence of a two-chain factor X precursor in the Golgi apparatus with the propeptide still attached, suggesting that internal proteolytic cleavage of the single-chain factor X precursor can precede proteolytic release of the propeptide. Also, the effect of the

Abbreviations used: ER, endoplasmic reticulum; DFP, di-isopropyl fluorophosphate; *p*-APMSF, *p*-amidinophenylmethanesulphonyl fluoride; E-64, *trans*-epoxysuccinyl-l-leucylamido-(4-guanidino)butane; DTT, dithiothreitol; endo H, endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.30); *N*-glycanase, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (EC 3.5.1.52).

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anticoagulant warfarin on factor X precursor trafficking in the secretory pathway has been studied. The results suggest that warfarin prevents exit of the factor X precursor from the ER, thus preventing the precursors from reaching the Golgi apparatus. It is demonstrated that the effect of the drug on factor X trafficking is associated with a large increase in the ER concentration of retained factor X precursors which, in contrast to the prothrombin precursors, appear to be stabilized in the ER compartment.

MATERIALS AND METHODS

Preparation of subcellular particles

All buffers used for preparation of subcellular particles contained 5 mM of the serine proteinase inhibitors di-isopropyl fluorophosphate (DFP) and benzamidine. Unless specified in the text, an inhibitor mix [leupeptin, 12.5 μg/ml; *p*-amidinophenylmethanesulphonyl fluoride (*p*-APMSF), 5 μg/ml; E-64, 2.5 μg/ml) was also added to all buffers. All procedures were carried out at 4 °C.

Liver microsomes and the Golgi apparatus

Liver microsomes were prepared and their separation into rough and smooth microsomes was carried out as described previously [3]. The Golgi apparatus was prepared as recently described in Stanton *et al.* [7] with some modifications. Portions of 300 g of perfused rat livers were minced and homogenized in 2 vol. of 37.5 mM-Tris/maleate, pH 7.0, containing 0.5 M-sucrose, 5 mM-$MgCl_2$, 1% dextran and 0.1% 2-mercaptoethanol (buffer A). The homogenate was centrifuged at 8000 *g* for 10 min in a fixed-angle JA-18 rotor (Beckman). Two-thirds of the pellet was removed from the top for further processing. The collected material was resuspended in 1 vol. of buffer A and centrifuged at 8000 *g* in a JS-13.1 Beckman swing-out rotor. The majority of the pellet was again removed and combined with buffer A in a ratio of 4:1 (w/v). The suspension was centrifuged through 37.5 mM-Tris/maleate, pH 7.0, containing 1.2 M-sucrose. The material sedimenting in the interface was collected and resuspended in 250 mM-sucrose/25 mM-imidazole/HCl, pH 7.2. The suspension was centrifuged at 10000 *g* for 10 min in a fixed-angle JA-18 rotor and the pellet was resuspended in 43.7% sucrose. A 2 ml portion of this suspension was overlayed with a sucrose gradient and centrifuged as described in [7] for isolation of the Golgi apparatus. The Golgi apparatus from the gradient was resuspended in 25 mM-imidazole/250 mM-sucrose, pH 7.2, and pelleted by centrifugation at 100000 *g* for 60 min. The Golgi pellets were stored at −70 °C until required for analysis.

Preparation of membrane fragments and luminal content of subcellular particles

Microsomes (5 mg/ml) and the Golgi apparatus (1 mg/ml) were resuspended with a Dounce homogenizer in 25 mM-imidazole/0.5% CHAPS, pH 7.2, containing all inhibitors specified above (buffer B). The suspension was shaken on ice for 30 min before it was centrifuged at 100000 *g* for 60 min. The supernatant containing the luminal content of the subcellular particles and the pellet containing the membrane fragments were separated. The pellets were surface-washed twice with buffer B before use in the experiments.

Vitamin K-specific ^{14}C-labelling of proteins in microsomal membrane fragments

Vitamin K-specific labelling was carried out basically as described in [14]. Microsomal membrane fragments (5 mg/ml) were resuspended in buffer B and incubated at 25 °C for 30 min in the presence of reduced vitamin K_1 (100 μg/ml), $H^{14}CO_3^-$ (25 μCi/ml), $MnCl_2$ (2 mM) and dithiothreitol (DTT) (5 mM). Following incubation, the suspension was centrifuged at 100000 *g* for 60 min to recover the membrane fragments. The supernatant was discarded and the pellets were surface-washed twice with buffer B before the membranes were solubilized in 25 mM-imidazole/0.5 M-KCl/1.5% Triton X-114, pH 7.2, containing all inhibitors specified above (buffer C).

Purification of membrane-associated ^{14}C-labelled precursors of prothrombin and factor X

Labelled microsomal membranes solubilized in buffer C were mixed in an Erlenmayer flask with anti-(rat prothrombin) antibodies coupled to CNBr-activated Sepharose 4B. The suspension was allowed to shake at 4 °C overnight before it was poured into an empty column that was connected to a u.v.-monitoring system. The void volume fraction was collected for isolation of the factor X precursor as described below. The anti-(rat prothrombin) immunoaffinity column was washed sequentially with 25 mM-imidazole/1% CHAPS/5 mM-DFP/5 mM-benzamidine, pH 7.2, containing 0.5 M-KCl, 1.0 M-KCl, and finally 2 M-NaBr. Bound prothrombin antigen was eluted from the column with 4 M-urea/0.5 M-KCl/1% CHAPS/0.1 M-acetate, pH 4.0. The isolated prothrombin precursors were precipitated by addition of 5 vol. of cold acetone to the urea buffer. The precipitate was washed with 10% trichloroacetic acid and then with diethyl ether/ethanol (1:1, v/v) before being subjected to two-dimensional immunoblotting as described below. The void volume fraction was mixed with anti-(rat factor X) antibodies coupled to CNBr-activated Sepharose 4B, and the suspension was again left shaking overnight at 4 °C. Isolation of the factor X precursors was carried out as described above for the prothrombin precursor.

Immunoprecipitation

Isolation of precursors of factor X present in the Golgi apparatus was achieved by immunoprecipitation with anti-(rat factor X) IgG coupled to Immunobead particles purchased from Bio-Rad, Richmond, CA, U.S.A. The Golgi apparatus and membrane fragments derived from it were dissolved in 25 mM-imidazole/1.5% Triton X-100/0.5 M-KCl, pH 7.2, containing all of the proteinase inhibitors specified above, before addition of the beads. Usually 100 μl of Immunobead particle suspension was added to 1 ml of protein solution. The particles were allowed to react with the antigen overnight at 4 °C. The particles were then isolated by centrifugation and washed three times with 25 mM-imidazole/0.5 M-NaCl/0.05% Triton X-100, pH 7.2. The antigen was released from the particles by boiling them in the SDS/PAGE running buffer containing 2% SDS and 5% mercaptoethanol.

SDS/PAGE and immunoblotting

One-dimensional SDS/PAGE was carried out in 10% gels according to Laemmli [15]. Two-dimensional SDS/PAGE was carried out by a modified version of the procedure of O'Farrell [16], as described in [17]. One-dimensional and two-dimensional immunoblots of the SDS/PAGE gels were obtained after transferring the electrophoresed proteins on to Immobilon P membranes as described [14]. The membranes were blocked with 5% non-fat milk proteins before reaction with the appropriate antiserum. Immunoreactive protein bands were visualized on the membranes after exposing them to a horseradish peroxidase-conjugated second antibody and 4-chloro-1-naphthol and H_2O_2 as described [14].

Immunodot-blotting

Quantification of prothrombin and factor X precursors in

detergent solutions of microsomes and Golgi apparatus, and of prothrombin and factor X zymogens in plasma, was carried out by immunodot-blotting as described in [14]. Samples of 1–2 μl were spotted on to nitrocellulose membranes which, subsequently, were blocked with 5% non-fat milk protein and reacted with the appropriate antiserum. Quantification was achieved by reacting the membranes with 0.1 μCi of ^{125}I-Protein A/ml and subjecting the spots to scintillation counting. Purified rat prothrombin and rat factor X were used as standards in this assay.

Glycosidase digestions

Golgi apparatus precursors of factor X were digested with endo H and *N*-glycanase when bound to the antibody–Immunobead particles used for isolation of the antigen. The antigen-containing particles were boiled in the presence of SDS and diluted as described [7] into the appropriate buffer used for digestion with the selected glycosidase. Incubations were carried out overnight at 37 °C, and then the reaction was stopped by addition of 10% trichloroacetic acid. Samples were prepared for SDS/PAGE after one wash in diethyl ether/ethanol (1:1, v/v) and solubilization by boiling the dried sample in the SDS/PAGE running buffer containing 2% SDS and 5% mercaptoethanol.

Peptide synthesis

The peptide ESLFIRREQANNILARVTRA, containing the -19 to $+1$ portion of the human factor X precursor [18], was synthesized by Bachem Fine Chemicals, Torrence, CA, U.S.A. The peptide, which contained the -16 to -1 propeptide portion of the factor X precursor, was guaranteed by the supplier to be >99% pure, based on h.p.l.c. analysis in three different chromatographic systems.

Immunology

Antisera against plasma rat prothrombin and factor X were produced in rabbits as described [14]. An antiserum directed against the factor X propeptide was obtained in rabbits by intradermal injections of the peptide ESLFIRREQANNILARVTRA coupled to keyhole limpet haemocyanin as described [3]. Immunoblotting confirmed that the anti-(factor X propeptide) antibodies did not recognize the factor X plasma zymogen but recognized the intracellular precursor of factor X, analogous to what we have described previously for anti-(factor X propeptide) antiserum raised in rabbits using a different procedure [14].

IgG was purified from antisera by 50% ammonium sulphate fractionation and chromatography on Affigel–protein A. IgG was eluted from the Affigel–Protein A column in 0.1 M-citrate, pH 3.0, and dialysed against the buffers used for coupling of IgG to solid supports. IgG from anti-prothrombin and -factor-X antisera were coupled as recommended by the supplier to CNBr-activated Sepharose (purchased from Sigma, St. Louis, MO, U.S.A.). IgG from the anti-(factor X) serum was also coupled to Immunobead particles (Bio-Rad). The procedure supplied with the product was followed in detail.

Materials

Endo H purified from *Streptomyces plicatus* was from ICN, Lisle, IL, U.S.A. Recombinant *N*-glycanase was from Genzyme, Boston, MA, U.S.A. CHAPS and Triton X-114 were purchased from Sigma. Affigel–Protein A was from Bio-Rad.

RESULTS

Immunoblotting of the factor X Golgi apparatus precursors revealed four immunoreactive bands, with estimated molecular masses of 74, 70, 50 and 21 kDa (Fig. 1*a*, lane 3). The apparent location of three proteins in the lumen and the membrane of the Golgi apparatus is shown in lanes 1 and 2 respectively. The 70 kDa protein was the dominant protein in the Golgi apparatus membrane preparation, and thus appears to be associated with the membrane (Fig. 1*a*, lane 2). On the other hand, the 74 kDa precursor appears to be located in the Golgi lumen, since it was the dominant protein found in the luminal extract of the Golgi apparatus (Fig. 1*a*, lane 1). The factor X precursor isolated from detergent-solubilized microsomes with anti-(factor X)–Immunobeads also appeared as a 70 kDa protein (Fig. 1*b*).

The 50 and 21 kDa immunoreactive proteins seen on the immunoblots in Fig. 1 were identified by the experiments shown in Figs. 2 and 3. In Fig. 2, rabbit IgG (lane 2) and rat plasma factor X (lane 3) were compared with the factor X immunoreactive proteins found in the Golgi apparatus (lane 1). As shown in lane 3, the heavy and light chains of rat plasma factor X

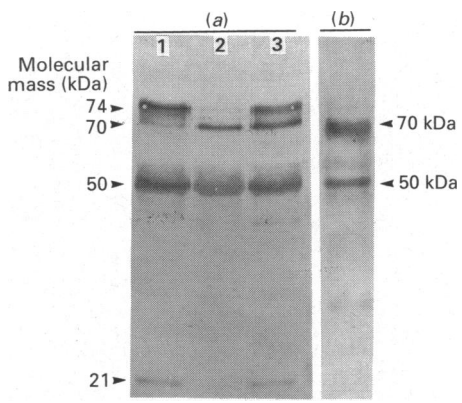

Fig. 1. Factor X precursors in the Golgi apparatus

Factor X precursors present in the Golgi apparatus (lane 3) and in a luminal extract (lane 1) and a membrane preparation (lane 2) of the Golgi apparatus were isolated with anti-(factor X)–Immunobeads and subjected to SDS/PAGE and immunoblotting as described in the Materials and methods section. The immunoblots were developed with an antiserum raised against rat plasma factor X. (*b*) Immunoblot of the factor X precursor isolated from rat liver microsomes with anti-(factor X)–Immunobeads.

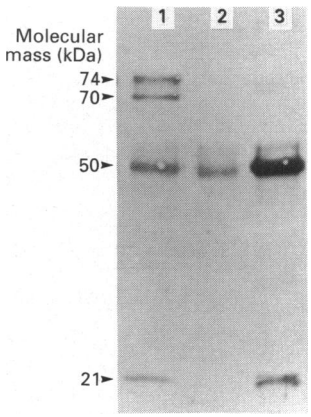

Fig. 2. Immunoblotting of plasma factor X and Golgi apparatus factor X precursors

Factor X antigens present in rat plasma (lane 3) and in the Golgi apparatus (lane 1) were isolated with anti-(factor X)–Immunobeads and subjected to SDS/PAGE and immunoblotting as described in the Materials and methods section. Lane 2 shows purified rabbit IgG. The immunoblot was developed with an antiserum raised against plasma factor X.

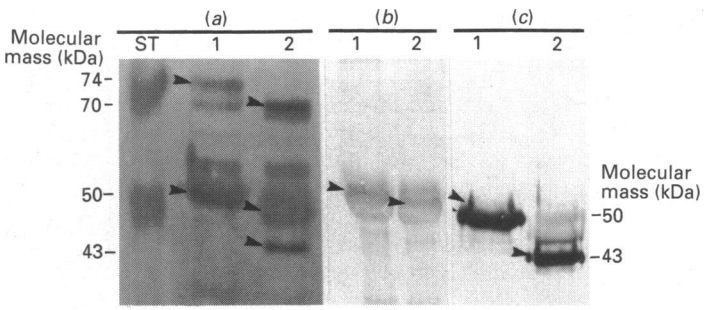

Fig. 3. *N*-Glycanase treatment of plasma factor X and Golgi apparatus factor X precursors

Factor X antigens present in rat plasma and the Golgi apparatus were isolated with anti-(factor X)–Immunobeads and digested with *N*-glycanase for determination of Asn-linked glycoproteins. Undigested and digested samples were subjected to SDS/PAGE and immunoblotting as described in the Materials and methods section. The immunoblots were developed with an antiserum raised against plasma factor X. Panels (*a*) and (*c*) show the Golgi apparatus precursors and the heavy chain of plasma factor X respectively before (lanes 1) and after (lanes 2) *N*-glycanase treatment. Panel (*b*) shows the heavy chain of rabbit IgG before (lane 1) and after (lane 2) *N*-glycanase treatment. Lane ST contains prestained standard proteins. The arrowheads indicate the positions on the immunoblots of immunoreactive bands.

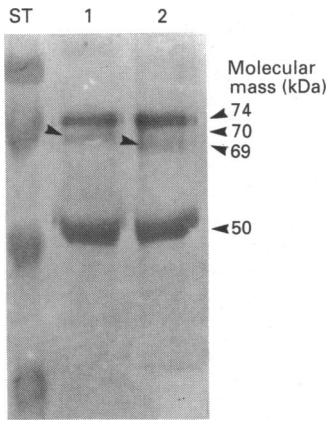

Fig. 4. Endo H treatment of Golgi apparatus factor X precursors

Factor X precursors present in the Golgi apparatus were isolated with anti-(factor X)–Immunobeads and digested with endo H. Undigested (lane 1) and digested (lane 2) samples were subjected to SDS/PAGE and immunoblotting as described in the Materials and methods section. The immunoblot was developed with an antiserum raised against plasma factor X. Lane ST contains prestained standard proteins. The arrowheads indicate the positions on the immunoblot of immunoreactive bands.

appeared on the immunoblot as 50 and 21 kDa protein bands respectively. The heavy chain of IgG showed the same molecular mass (50 kDa) as the heavy chain of factor X (lane 2). However, the second antibody did not recognize the light chain of IgG (lane 2). The results from this experiment suggested that a two-chain form of the factor X precursor was present in the Golgi apparatus preparation, together with the 70 and 74 kDa single-chain factor X precursors, and that IgG released from the anti-(factor X)–Immunobeads used for isolation of the antigens was a possible contaminant in our preparation.

The experiment shown in Fig. 3 further supports these conclusions. Digestion of plasma factor X with *N*-glycanase converted

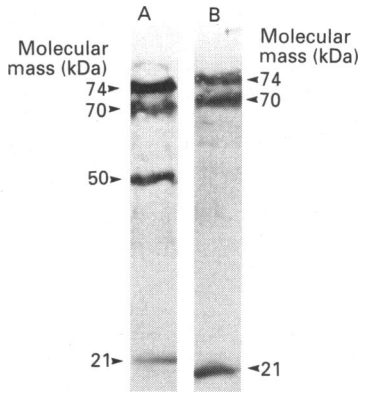

Fig. 5. Identification of the factor X propeptide in Golgi apparatus factor X precursors

Golgi apparatus factor X precursors were isolated by immunoaffinity chromatography as described in the Materials and methods section. Aliquots of the precursor preparation were subjected to SDS/PAGE and immunoblotting with an antiserum raised against the factor X propeptide (lane B) or plasma factor X (lane A).

the 50 kDa heavy chain of factor X (Fig. 3*c*, lane 1) into a 43 kDa protein (lane 2), demonstrating the presence of Asn-linked carbohydrates on the heavy chain of rat factor X. The light chain of factor X was insensitive to *N*-glycanase treatment (results not shown). Fig. 3(*b*) demonstrates that the apparent molecular mass of the heavy chain of IgG was very little affected by *N*-glycanase treatment. Fig. 3(*a*) shows that some of the 50 kDa immunoreactive protein from the Golgi apparatus factor X precursor preparation (lane 1) also shifted to a 43 kDa position after *N*-glycanase treatment (lane 2). However, the 50 kDa protein also produced a new band with a much smaller shift in molecular mass after *N*-glycanase treatment (lane 2), and this protein behaved as the heavy chain of IgG (compare Figs. 3*a* and 3*b*). *N*-Glycanase treatment of the Golgi apparatus factor X precursors also provided evidence that the 74 and 70 kDa single-chain precursors are *N*-linked glycoproteins. Following *N*-glycanase digestion the largest shift in molecular mass was seen for the 74 kDa precursor, which suggested that this was further along the processing pathway than the 70 kDa precursor. This was indeed confirmed by endo H digestion of the Golgi apparatus factor X precursors. Only the 70 kDa precursor (Fig. 4, lane 1) shifted to a lower molecular mass after treatment with endo H (lane 2), which identified the protein as one bearing high-mannose sugars. Also shown in Fig. 4 is the finding that the heavy chain of factor X (50 kDa) appearing in the Golgi apparatus preparation was insensitive to endo H. Thus the endoproteinase responsible for cleavage of the single-chain factor X precursor into a two-chain precursor uses a late-processing form of the precursor as substrate.

In contrast to earlier findings with late-processing forms of prothrombin [7], we could not identify factor X precursors in our Golgi apparatus preparations that were devoid of the propeptide. As shown in Fig. 5 (lane B), an antiserum raised against the factor X propeptide recognized the 74 and 70 kDa single-chain factor X precursors, and also the 21 kDa light chain of factor X. No immunoreaction was seen, however, with the 50 kDa heavy chain of factor X (Fig. 5, lane B). On the other hand, an antiserum raised against plasma factor X also recognized the heavy chain (lane A). Since the second antibody did not recognize the 50 kDa heavy chain of IgG in lane B, this demonstrated that no contaminating IgG was present in this preparation of the factor X precursors, which had been purified from the Golgi apparatus by immunoaffinity chromatography. The experiment

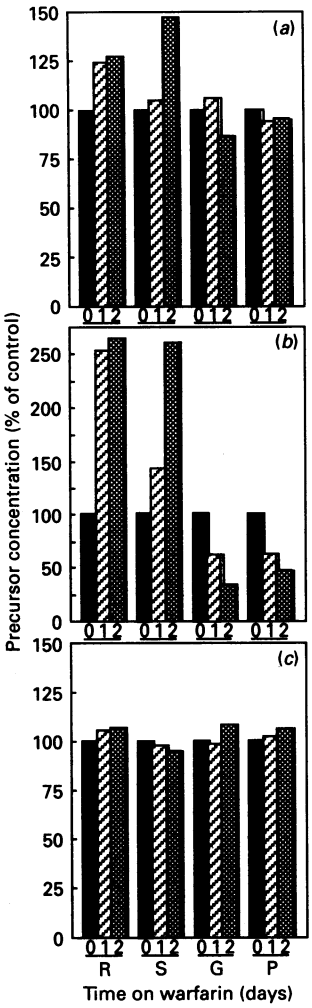

Fig. 6. Effect of warfarin on subcellular precursor concentrations of factor X, prothrombin and albumin

Groups of rat were injected with warfarin (30 mg/kg) or saline (control) on day 0 and killed on days 1 and 2 for isolation of rough (R) and smooth (S) microsomes, the Golgi apparatus (G) and plasma (P). The antigen concentrations of prothrombin (*a*), factor X (*b*) and albumin (*c*) were determined by immunodot-blotting using antisera raised against the purified plasma proteins as described in the Materials and methods section. The concentrations measured in warfarin-treated animals are presented as percentages of the concentrations measured in saline controls on day 0 (■), day 1(▨) and day 2 (▩). Data are the means of three parallel determinations differing by < 10%.

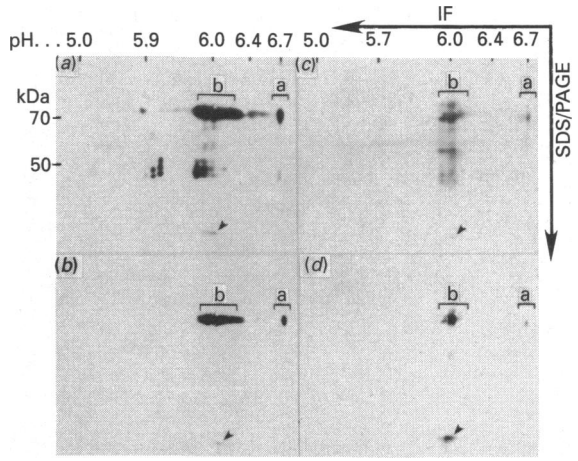

Fig. 7. Two-dimensional immunoblotting of microsomal factor X precursors from normal and warfarin-treated rats

Liver microsomal membranes were prepared from normal and warfarin-treated rats and used for vitamin K-dependent ^{14}C-labelling of membrane-associated proteins as described in the Materials and methods section. Equal amounts of labelled membranes from normal and warfarin-treated rats (200 mg of protein) were used as starting material for purification of the factor X precursors from normal and warfarin membranes respectively. Aliquots of the isolated precursor pools from normal (*c*) and warfarin (*a*) membranes were subjected to two-dimensional immunoblotting and the immunoblots were developed with an antiserum raised against plasma factor X. Two different pools of factor X precursors, labelled a and b on the Figure and defined by the brackets, were separated in the direction of isoelectric focussing (IF). Panels (*b*) and (*d*) are autoradiograms of the immunoblots shown in (*a*) and (*c*) respectively. The arrowheads indicate the position on the immunoblots of the light chain of factor X.

demonstrated that intracellular conversion of a single-chain factor X precursor to a two-chain molecule had occurred without cleavage of the propeptide.

In order to reach the site in the Golgi apparatus for endoproteolytic processing, the factor X precursors must exit the ER and move through the cis-, medial and trans-Golgi compartments [19]. Trafficking of the factor X precursors in the secretory pathway is known to be affected by the anticoagulant drug warfarin [3,20], and the next set of experiments was designed to localize the site in the secretory pathway where warfarin interferes with factor X precursor trafficking. Groups of rats were given warfarin (30 mg/kg) on day 0 and killed on day 1 or day 2. Controls were saline-injected rats killed on the same days. Rough and smooth microsomes and the Golgi apparatus were isolated from livers for determination of factor X, prothrombin and albumin precursor concentrations. These concentrations were also determined in plasma collected from the rats. Fig. 6 presents the precursor and plasma concentrations as percentages of those in the saline controls. The drug had the greatest effect on the factor X precursor concentration, which had increased to 265% in rough and smooth microsomes and decreased to 35% in the Golgi apparatus at 2 days after administration of the drug (Fig. 6*b*). Also, the plasma concentration of factor X was decreased to 40% by day 2 (Fig. 6*b*). Consistent with previous findings [3], warfarin increased the microsomal concentration of the prothrombin precursor, but not to the same extent as the factor X concentration (Fig. 6*a*). A small but significant decrease to 85% ($P < 0.02$) in the Golgi apparatus prothrombin precursor concentration was also measured on day 2, but no significant change was seen in plasma concentrations (Fig. 6*a*). Warfarin did not affect the albumin precursor concentrations measured in the subcellular particles (Fig. 6*c*). These data strongly suggest that warfarin decreases the flux of factor X precursors from the ER to the Golgi apparatus, hence blocking further processing of the retained proteins.

Additional experiments were conducted to investigate the factor X precursors that were retained in the ER as a result of warfarin administration. Since warfarin inhibits γ-carboxylation of the precursors, we focused on the membrane-associated precursors which have been shown previously to be substrates for the membrane-bound carboxylase *in vitro* [14]. The membrane fragments of microsomes from warfarin and control rats were prepared, the associated precursors were [^{14}C]-γ-carboxylated and the labelled prothrombin and factor X precursors were isolated by immunoaffinity chromatography as described in the legend to Fig. 7. Figs. 7(*a*) and 7(*a*) show two-dimensional immunoblots of the membrane-associated factor X precursors

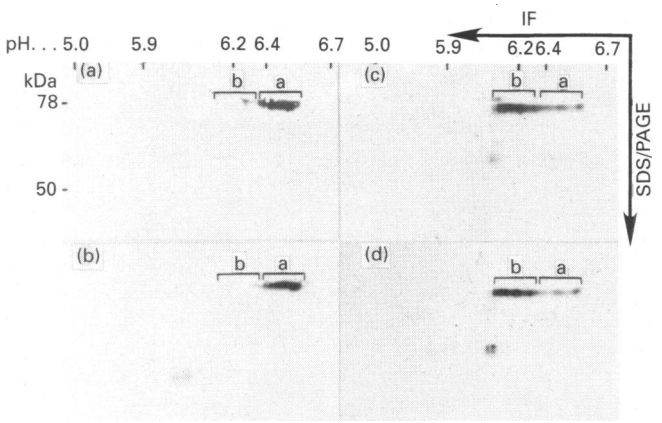

Fig. 8. Two-dimensional immunoblotting of microsomal prothrombin precursors from normal and warfarin-treated rats

Microsomal prothrombin precursors were isolated from the same ^{14}C-labelled membranes as used for isolation of the membrane-associated factor X precursors (see legend to Fig. 7 and the Materials and methods section). Aliquots of the isolated precursor pools from normal (*c*) and warfarin (*a*) membranes were subjected to two-dimensional immunoblotting, and the immunoblots were developed with an antiserum against plasma prothrombin. Panels (*b*) and (*d*) are autoradiograms of the immunoblots shown in (*a*) and (*c*) respectively. Two different pools of prothrombin precursors (labelled a and b), separating in the direction of isoelectric focusing (IF), were defined as shown by the brackets.

Table 1. Variation in the membrane concentration of factor X and prothrombin precursors

Pools a and b, as defined in Figs. 7 and 8, of the factor X and prothrombin precursors, were estimated quantitatively by densitometric laser scanning and integration of the stained areas. The integrated area of pool a (in Figs. 7*c* and 8*c*) from normal rats was used as a unit for the estimation of relative changes in the prothrombin and factor X precursor concentrations respectively.

	Area (units)			
	Prothrombin		Factor X	
ER membrane	Pool a	Pool b	Pool a	Pool b
Control rats	1.0	3.0	1.0	6.2
Warfarin-treated rats	3.7	0	10.1	60.4

isolated from warfarin and control microsomes respectively. In addition to their pI values and molecular masses, the immunoblot also gave a quantitative measure of the size of the precursor pool associated with the membrane (see legend to Fig. 7). Two pools of factor X precursors differing in pI, labelled a and b in Fig. 7, were apparent on the immunoblot from control rats (Fig. 7*c*) as well as on the immunoblot from warfarin-treated rats (Fig. 7*a*). The experiment demonstrated a large increase in the total membrane-associated factor X pool when rats were treated with warfarin, and there was a parallel increase in pools a and b (compare Figs. 7*c* and 7*a*). Figs 7(*b*) and 7(*d*) show autoradiograms of the two-dimensional immunoblots of Fig. 7(*a*) and Fig. 7(*c*) respectively. The autoradiograms were identical to the respective immunoblots, except for some immunoreactive spots with molecular mass around 50 kDa seen on the immunoblot in Fig. 7(*a*). These spots probably represent minor quantities of the heavy chain of factor X present in our preparation of the microsomal factor X precursor. The position on the immunoblot of the ^{14}C-labelled light chain containing the Gla region of factor X is indicated by the arrowheads in Fig. 7.

An identical experiment was carried out with the membrane-associated prothrombin precursors, and the two-dimensional immunoblots are shown in Figs. 8(*a*) and 8(*c*) respectively. The prothrombin precursors became a more basic group of proteins upon treatment of rats with warfarin (compare Figs. 8*a* and 8*c*). In addition, laser scanning and integration of the immunoreactive areas on the immunoblots showed no significant difference, indicating that the total pool of membrane-associated prothrombin precursors had not changed in quantity as a result of warfarin treatment of the rat in contrast with what was seen for the factor X precursors. Figs. 8(*b*) and 8(*d*) show autoradiograms of the immunoblots of Figs. 8(*a*) and 8(*c*) respectively. As seen for the factor X precursors, the autoradiograms of the prothrombin precursors were identical to the immunoblots, suggesting that all of the membrane-associated prothrombin and factor X precursors could be used as substrates for the carboxylase *in vitro*. We also defined two different pools (pools a and b) for the prothrombin precursors with different pI values (Fig. 8). Table 1 shows the variation in the prothrombin and factor X precursor concentrations in pools a and b from warfarin-treated and control rats when the densities of the stained areas a and b shown in Figs. 7 and 8 were estimated by laser scanning of the membranes. The data demonstrate (Table 1) that warfarin resulted in a 10-fold increase in the membrane concentration of factor X precursors. The increase was the same for precursors present in pools a and b. Warfarin resulted in a 3.7-fold increase in the prothrombin precursors present in the more basic pool a on the membrane but, in great contrast to what was found for the factor X precursors, the more acidic pool b of the prothrombin precursors was barely detectable on the immunoblot (see Table 1). These data suggest that warfarin treatment of the rats replaced the more negatively charged prothrombin precursors seen in controls (pool b) with precursors that were less negatively charged (pool a). Since the autoradiogram of the ^{14}C-labelled prothrombin precursors from warfarin-treated rats did not extend into the pool b region (Fig. 8*b*), the experiment also demonstrated that γ-carboxylation *in vitro* of the precursor pool isolated from warfarin-treated rats could not produce the more negatively charged precursors found in control rats.

DISCUSSION

This paper demonstrates that the intracellular precursors of clotting factor X originate as a 70 kDa protein in the ER and mature into a single-chain 74 kDa protein in the trans-Golgi apparatus. Factor X is a two-chain zymogen in blood, which necessitates intracellular proteolytic processing of the single-chain precursor before the clotting factor leaves the hepatocyte via the constitutive pathway [19]. A similar situation exists for protein C, which has been studied by Foster *et al.* [12,13]. Their transfection experiments have provided data which strongly support the hypothesis that furin, the KEX2-like gene product, is the endoproteinase that cleaves single-chain protein C into a two-chain form. Our Golgi apparatus preparation contained single-chain factor X precursors in early and late processing stages, as well as a two-chain precursor of factor X. The data demonstrate that the two-chain factor X precursor had been derived from a late-processing form of single-chain factor X, indicating that the site for proteolysis is further along the secretory pathway than the site at which endo H sensitivity is lost (the late medial Golgi, the trans-Golgi or the trans-Golgi network). Furin action takes place in the trans-Golgi [8]. Thus the subcellular location of the endoproteinase involved in factor X intra-chain cleavage is consistent with the location of the

enzyme which removes the prothrombin propeptide [7]. However, we were unable to demonstrate factor X precursor forms in the Golgi apparatus which were devoid of the propeptide, which raises questions about whether or not the factor X propeptide is indeed released in the trans-Golgi compartment. In this context, it is interesting to note that factor X is the only clotting factor which does not have a dibasic sequence at the propeptide cleavage site [20] which is proposed to be a requirement for proteolysis by the KEX2 gene family of endoproteinases.

Our data on subcellular precursor concentrations indicate that the anticoagulant drug warfarin blocks the exit of the factor X precursors from the ER, preventing them from reaching the Golgi apparatus for further processing. Since warfarin inhibits the vitamin K cycle in liver [21], this suggests a link between γ-carboxylation of the precursors and their exit from the ER. Using a different experimental approach from that described here, Tollesrud *et al.* [22] have proposed a similar model. This concept would indeed be consistent with a more general view of the ER as a sorting point in the secretory pathway for incorrectly and correctly folded proteins [23], and also implies a possible involvement of ER resident proteins other than the vitamin K-dependent carboxylase in the process of retention. Folding of vitamin K-dependent proteins is indeed affected by γ-carboxylation of the Gla region [24], and may be part of the retention/exit signal for the precursors. Since incompletely γ-carboxylated vitamin K-dependent clotting factors are found in human and bovine plasma after warfarin administration [25], a bypass mechanism for exit from the ER must also exist in certain species.

Further studies were conducted to investigate the ER-retained factor X and prothrombin precursors in warfarin-treated rats. Warfarin caused the membrane-associated factor X precursor concentration to increase 10-fold; in contrast, the prothrombin precursor concentrations remained unchanged. This finding is consistent with previous published data [14,26]. Two pools of factor X precursors with different pI values were demonstrated to be associated with the membranes from normal rats. Warfarin resulted in an increase in both factor X precursor pools, demonstrating a major difference between prothrombin and factor X precursors: the more negatively charged prothrombin pool of precursors was eliminated from the membrane after warfarin treatment. These data suggest the existence in the ER of a stabilizing mechanism for the factor X precursors that is not operative for all vitamin K-dependent precursor proteins.

We have previously presented data which indicate that the extra negative charges acquired by the prothrombin precursors in normal rats is the result of γ-carboxylation [7]. It appears from our present data that a stop in γ-carboxylation of the Gla region in the ER results in elimination of the more negatively charged prothrombin precursors, possibly by intracellular degradation, as has also been suggested by others [22].

Finally, the data demonstrate that γ-carboxylation *in vitro* of prothrombin precursors associated with the ER membrane in warfarin-treated rats cannot restore the more negatively charged pool of prothrombin precursors found in the ER membrane from normal rats. Thus continued γ-carboxylation of the Gla region is halted *in vitro* even in the presence of excess reduced cofactor for the vitamin K-dependent carboxylase. Since the Gla region is completed *in vivo* when sufficient vitamin K cofactor is available to the carboxylase [27], the data demonstrate that a component necessary for continued γ-carboxylation of the Gla region is lost by disruption of the hepatocyte and the secretory pathway. Thus an understanding of vitamin K-dependent γ-carboxylation, as a post-ribosomal modification of secretory proteins, appears to also involve an understanding of how other cellular processes are coupled to these events.

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